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Wnt11/*Fgfr1b* cross-talk modulates the fate of cells in palate development

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Abstract

Various cellular and molecular events underlie the elevation and fusion of the developing palate that occurs during embryonic development. This includes convergent extension, where the medial edge epithelium is intercalated into the midline epithelial seam. We examined the expression patterns of *Wnt11* and *Fgfr1b* – which are believed to be key factors in convergent extension – in mouse palate development. Wnt-11 overexpression and beads soaked in SU5402 (an Fgfr1 inhibitor) were employed in *in vitro* organ cultures. The results suggested that interactions between *Wnt11* and *Fgfr1b* are important in modulating cellular events such as cell proliferation for growth and apoptosis for fusion. Moreover, the *Wnt11* siRNA results showed that *Wnt11*-induced apoptosis was necessary for palatal fusion. In summary, *Fgfr1b* induces cell proliferation in the developing palate mesenchyme so that the palate grows and contacts each palatal shelf, with negative feedback of Fgfs triggered by excessive cell proliferation then inhibiting the expression of *Fgfr1b* and activating the expression of *Wnt11* to fuse each palate by activating apoptosis.

Keywords: *Wnt11*; *Fgfr1b*; Palatogenesis; Palatal growth; Palatal fusion; Cell proliferation; Apoptosis; z-VAD-fmk; *Wnt11* siRNA

Introduction

Various cellular and molecular mechanisms underlie the elevation and fusion of the palate shelves that are integral to normal mammalian palatogenesis (Taniguchi et al., 1995; Gritli-Linde, 2007; Lee et al., 2007). Two palatal shelves grow from the internal surfaces of the maxillary primordia once the development of the secondary palate is initiated, and at embryonic day 13.5 (E13.5) in mouse they appear vertically on each side of the tongue, elevating to a horizontal position above the tongue at E14.0 (Ferguson, 1987). From E14.0 to E14.5 the palatal shelves make contact and start the fusion necessary for correct morphogenesis (Johnston and Bronsky, 1995), and by E14.5 they are fused with one another from the middle region to the anterior and posterior regions to transform the medial edge epithelium (MEE) into the midline epithelial seam (MES) (Martýnez-Alvarez et al., 2000; Chou et al., 2004). Then, at E15.5, the MES is completely removed from the fused palate. The fate of the MEE, which is known to form the MES upon palatal shelf fusion, is still debated. Previous studies have revealed that the cleft secondary palate originates from a failure of signaling molecules and their receptors to control proliferation and fusion (Thyagarajan et al., 2003; Yu et al., 2005; Chai and Maxson, 2006). Several mutation studies have revealed that a cleft palate results from intrinsic disruptions to the cellular and molecular events controlling palatal shelf growth, elevation, or fusion (Gritli-Linde, 2007). *Fgf10*$^{-/-}$ mice show cleft palate because of abnormal fusion between the palatal shelves and the tongue or mandible (Alappat et al., 2005). Moreover, several defects in craniofacial development, including cleft palate, have been observed in skeletal preparations of *Wnt1* and *Fgfr1* mutant newborns (Trokovic et al., 2003).

The sequence of morphological changes in palatogenesis can be considered similar to processes in other types of organogenesis, such as body axis formation, which is also called convergent extension (Tudela et al., 2002). Convergent extension is

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broadly defined as the process in which a tissue narrows along one axis and lengthens in the perpendicular axis and is critical to the formation of the vertebrate body axis (Keller et al., 1985; Wallingford et al., 2002). Signaling molecules such as *Wnt11* and *Fgfr1* are closely associated with convergent extension movement during other types of organogenesis, including mouse kidney development (Majumdar et al., 2003; Chi et al., 2004).

The *Wnt* gene family encodes a conserved class of secreted signaling molecules and is considered to be one of the major gene families essential for correct embryonic pattern and organogenesis (Wodarz and Nusse, 1998; Maye et al., 2004; Yue et al., 2006). *Wnt11* promotes the calcium pathway (Du et al., 1995; He et al., 1997; Maye et al., 2004; Nelson and Nusse, 2004) that is involved in cell adhesion (Torres et al., 1996) and inhibition of the canonical Wnt signaling pathway (Ishitani et al., 2003; Widelitz, 2005). *Wnt11* is also essential for the canonical Wnt pathway activity in dorsal axis formation (Tao et al., 2005). However, the expression pattern and function of *Wnt11* in palatogenesis have not been elucidated.

In this study, we focused on the interaction with the Wnt pathway since FGF/Wnt cross-talk is emerging as an important nexus in the regulation of various biological processes. Wnt signaling is reported to be closely linked to Fgf signaling in the development of other organs, such as controlling the elongation of digits and tip formation (Sanz-Ezquerro and Tickle, 2003). Therefore, it is certain that Fgf expression is closely related to Wnt signaling during tip formation, which represents genetic evidence of a molecular link between the Wnt and Fgf signaling pathways in the patterning of the nascent mesoderm. Moreover, members of the Fgf family regulate Snail, which is the key modulator of cell survival during palate development (Rice et al., 2004) and a regulator of epithelial–mesenchymal transformation (EMT) during gastrulation (Carver et al., 2001).

To investigate the molecular interactions between *Wnt11* and *Fgfr1b*, we employed an overexpression method as a gain of function and treatment with pharmacological inhibitors as a loss of function with an *in vitro* palate culture system. Moreover, we treated *Wnt11* small interfering RNA (siRNA) during *in vitro* palate culture to confirm the functional significance of *Wnt11* in regulating palatal fusion. This study revealed the precise functions of cellular modulations controlled by the expression of specific genes, with the findings elucidating the unique genetic encoding underlying palatal development.

Materials and methods

All experiments complied with the guidelines of the Intramural Animal Use and Care Committee, College of Dentistry, Yonsei University.

Animals

Adult ICR mice were housed in a temperature-controlled room (22 °C) under artificial illumination (lights on from 05:00 to 17:00 h) and at 55% relative humidity with access to food and water *ad libitum*. The embryos were obtained from time-mated pregnant mice. E0 was designated as the day on which the presence of a vaginal plug was confirmed. Embryos at developmental stage E13.5–E15.5 were investigated.

In vitro organ culture

The palatal shelves were isolated from E13.5 mouse maxillae and cultured in medium without fetal bovine serum at 37 °C and 5% CO_2 for 48 h using a slight modification of the culture method reported by Trowell (Taya et al., 1999). The culture medium (DMEM/F12, Gibco) was supplemented with 20 μg/ml ascorbic acid (Sigma) and 1% penicillin/streptomycin and was renewed every 24 h. Tissues were then fixed and processed for *in situ* hybridizations, TUNEL assay, and immunohistochemistry. At least 10 specimens were examined in each experiment.

Whole-mount in situ hybridization

Specimens were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS). For *in situ* hybridization the specimens were treated with 20 μg/ml proteinase K for 3 min at room temperature (Kim et al., 2005). Antisense RNA probes were labeled with digoxigenin (Roche). After *in situ* hybridization, the specimens were cryosectioned at a thickness of 25 μm. At least five specimens were examined in each stage.

Expression constructs

pEGFP-N1 (Clontech) was used for tagging *Wnt11* protein with EGFP. Plasmid DNA was purified using a plasmid purification kit (Qiagen) and dissolved in TE [10 mM Tris–HCl (pH 8.0) and 0.25 mM EDTA].

Electroporation of organ cultures

The Wnt-11 expression construct (1 μg/μl) in PBS was injected into both palatal epithelium and mesenchyme using a microcapillary needle, and 20-ms current pulses were applied using an electroporator. Before the injection, fast green (diluted 1:10,000; Sigma) was added to the DNA solution for visualization within the tissue. At least 10 specimens were examined in each experiment.

Bead implantation

AG-1X2 (Bio-Rad Laboratories) formate-derived beads were incubated in 10 mM SU5402 (Calbiochem) for 1 h at room temperature. Beads were implanted on the palatal shelf at E13.5. After 48 h of culture *in vitro*, the palate was used for whole-mount *in situ* hybridization, TUNEL assay, and immunohistochemistry. At least 10 specimens were examined in each experiment.

Immunohistochemistry and TUNEL assay

The specimens were embedded in OCT compound using conventional methods. Sections (7 μm thick) of the specimens were incubated at 4 °C overnight with the primary mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA, NeoMarkers). After washing with PBS, the specimens were allowed to react with biotinylated goat antimouse immunoglobulins and streptavidin peroxidase at room temperature for two consecutive 10-minute incubations. Finally, the specimens were visualized using a 3,3′-diaminobenzidine (DAB) reagent kit (Zymed). A TUNEL assay was performed using an *in situ* cell apoptosis detection kit (Trevigen) following the manufacturer's instructions. The 7-μm-thick sections were treated with proteinase K [in 10 mM Tris–HCl (pH 8.0)] at a concentration of 20 μg/ml for 15–20 min at room temperature. The samples were then incubated with the labeling reaction mixture at 37 °C for 1 h and streptavidin–HRP solution for 10 min at room temperature. DAB was used as a substrate solution to detect the sites of *in situ* apoptosis under a light microscope. At least 10 specimens were examined in each experiment.

In vitro organ culture with the apoptosis inhibitor z-VAD-fmk

In the experimental treatments, media were supplemented with 25 μM, 50 μM, or 100 μM z-VAD-fmk (FK-009 z-VAD (OMe)-CH2F *O*-methylated, Enzyme Systems Products) in DMSO (Sigma, USA) for 2 days. At least 10

explants for each concentration of the z-VAD-fmk-treated group and the control group were observed in each experiment.

Small interfering RNA treatment

A 500 nM siRNA solution was prepared as follows. A siRNA stock (50 μM) was diluted with DMEM/F12 medium containing transfection reagent (siPORT™*NeoFX*™, Ambion) and then incubated for 10 min at room temperature. Scrambled control siRNA (Silencer® Negative-Control siRNA, Ambion) and *Wnt11* siRNA (Silencer® Predesigned siRNA; siRNA ID 65306, Ambion) were applied at a final concentration of 500 nM (Shiomi et al., 2006; Nakajima et al., 2007). siRNA solutions (2 ml) were applied to each culture plate containing five sets of paired palatal shelves. The palatal fusion process was confirmed by characterizing the MES region after culturing for 72 h from E13.5.

Real-time quantitative PCR

Total RNA was isolated from the cultured palate using the RNeasy® Mini Kit (Qiagen). For cDNA synthesis, reverse transcription was performed using M-MuLV reverse transcriptase (New England BioLabs). Real-time quantitative PCR (RT-qPCR) was performed using Thermal Cycler Dice™ Real Time System (TP800, Takara) with SYBR Premix EX Taq™ (Takara). The amplification program consisted of 45 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 20 s, and extension at 72 °C for 20 s. The results of RT-qPCR for each sample were normalized *β-actin*. The data were analyzed with the Thermal Cycler Dice™ Real Time System analysis software (Takara). The results were expressed as normalized ratios. The primer sequences of the genes are as follows: *β-actin*, 5′-ATTGCCGACAGGATGCAGA-3′ (forward) and 5′-GAGTACTTGCGCTCAGGAGGA-3′ (reverse); *Wnt11* (designed by Takara Bio), 5′-GGATCCCAAGCCAATAAACTGATG-3′ (forward) and 5′-TTGAGGTCAGCAGCCACGTC-3′ (reverse); and *Fgfr1b*, 5′-GGGAATTAA-TAGCTCGGATGA-3′ (forward) and 5′-ACGCAGACTGGTTAGCTTCA-3′ (reverse). Data were expressed as mean±SD. The mean expression levels were compared between the experimental and control groups using ANOVA (SPSS 10.0), with a probability value of $P<0.05$ (Tukey's HSD test) being considered statistically significant.

Results

Expression patterns of Wnt11 and Fgfr1b in the developing mouse palate

Wnt11 expression was examined by *in situ* hybridization at E13.5, E13.75, E14.0, and E14.5 (Figs. 1A–H). Each specimen was frontally sectioned at the level of the black dotted lines in the figure (Figs. 1A–P). At E13.5, *Wnt11* was not expressed in the anterior to posterior regions of the secondary palate (Fig. 1A) or in either the epithelium or mesenchyme of the palatal shelf (Fig. 1E). However, at E13.75 the palatal shelves were elevated above the tongue but were still not in contact with each other, and *Wnt11* was strongly expressed in the MEE region of the palatal shelves (Fig. 1B) and on the nasal to oral sides of the palatal shelf epithelium (Fig. 1F). At E14.0, the middle parts of the palatal shelves began to make contact with each other. *Wnt11* was expressed in the epithelium of the MEE region of both the contacted and noncontacted parts of the palatal shelves (Fig. 1C), and the expression was strong in the MES and on the nasal and oral sides of the fusion region in the palatal epithelium (Fig. 1G). At E14.5, *Wnt11* expression was observed along the entire fusion line (Fig. 1D) and was strong on the oral and nasal sides of the palatal epithelium (Fig. 1H) but weak in the MES region.

At E13.5, *Fgfr1b* was expressed from the anterior to middle regions of the mouse secondary palate (Fig. 1I). Frontal sections showed that *Fgfr1b* was expressed in the palatal mesenchyme of the MEE region (Fig. 1M). The expression pattern of *Fgfr1b* did not change after elevation and fusion (Figs. 1J–L). At E13.75, *Fgfr1b* was expressed from the anterior to middle regions of the palatal shelves (Fig. 1J) and in the palatal mesenchyme, similar to E13.5 (Fig. 1N). At E14.0, *Fgfr1b* was expressed from the anterior to middle regions of the secondary palate (Fig. 1K). Interestingly, *Fgfr1b* was not expressed at all in the region of palatal contact of both the epithelium and mesenchyme (Fig. 1O). At E14.5, *Fgfr1b* was expressed throughout the anterior and posterior regions of the secondary palate (Fig. 1L). Frontal sections revealed strong *Fgfr1b* expression in the mesenchymal region (Fig. 1P).

Alteration of gene expression patterns after applying SU5402-soaked beads and Wnt11 overexpression

In order to compare the molecular regulation between *Wnt11* and *Fgfr1b*, Wnt-11 was artificially overexpressed in the developing secondary palate, and beads soaked with the Fgfr1 inhibitor SU5402 were implanted in *in vitro* organ cultures at E13.5 for 48 h (Figs. 2A–D). In culture, the two palatal shelves grew toward each other to fill the initial gap of 0.04 mm to establish palatal contact. In this stage, the MEE region also grew and migrated with the palatal shelves. This made it difficult to inject the expression construct of Wnt-11 and to implant SU5402 beads exactly at the MEE position. *Wnt11* was expressed only in the palatal epithelium, in the MEE and MES regions. Therefore, we attempted to induce Wnt-11 overexpression only in the palatal epithelium, avoiding the mesenchyme. However, it was technically impossible to microinject the Wnt-11 construct only in the epithelium, and hence the construct was injected into both the palatal epithelium and mesenchyme.

Wnt-11 was overexpressed, as shown within the red dotted circle in Fig. 2A. Wnt-11 was successfully transfected, as judged by GFP expression (Fig. 2A′). Moreover, RT-qPCR was performed to confirm whether the Wnt-11 was successfully transfected (Fig. 2E). The level of *Wnt11* differed significantly between the Wnt-11 overexpressed and control groups. Relative to the control on the right side (Fig. 2A), Wnt-11 overexpression led to the inhibition of *Fgfr1b* expression at the anterior to middle regions after 48 h. The expression of *Fgfr1b* was weaker on the Wnt-11 overexpressed side than on the control side (Fig. 2C). Moreover, the expression of *Wnt11* was upregulated at 48 h after implanting SU5402-soaked beads (Fig. 2B), and SU5402 strongly induced *Wnt11* expression on the oral side of the palatal epithelium and weakly in the underlying mesenchyme (Fig. 2D). These results indicate that *Wnt11* is related to the Fgfr1 pathway.

Alterations to cellular events after treatment with SU5402-soaked beads and Wnt11 overexpression

Cell death was examined in control and Wnt-11 overexpressed specimens (Fig. 3A). Significantly, many apoptotic cells were

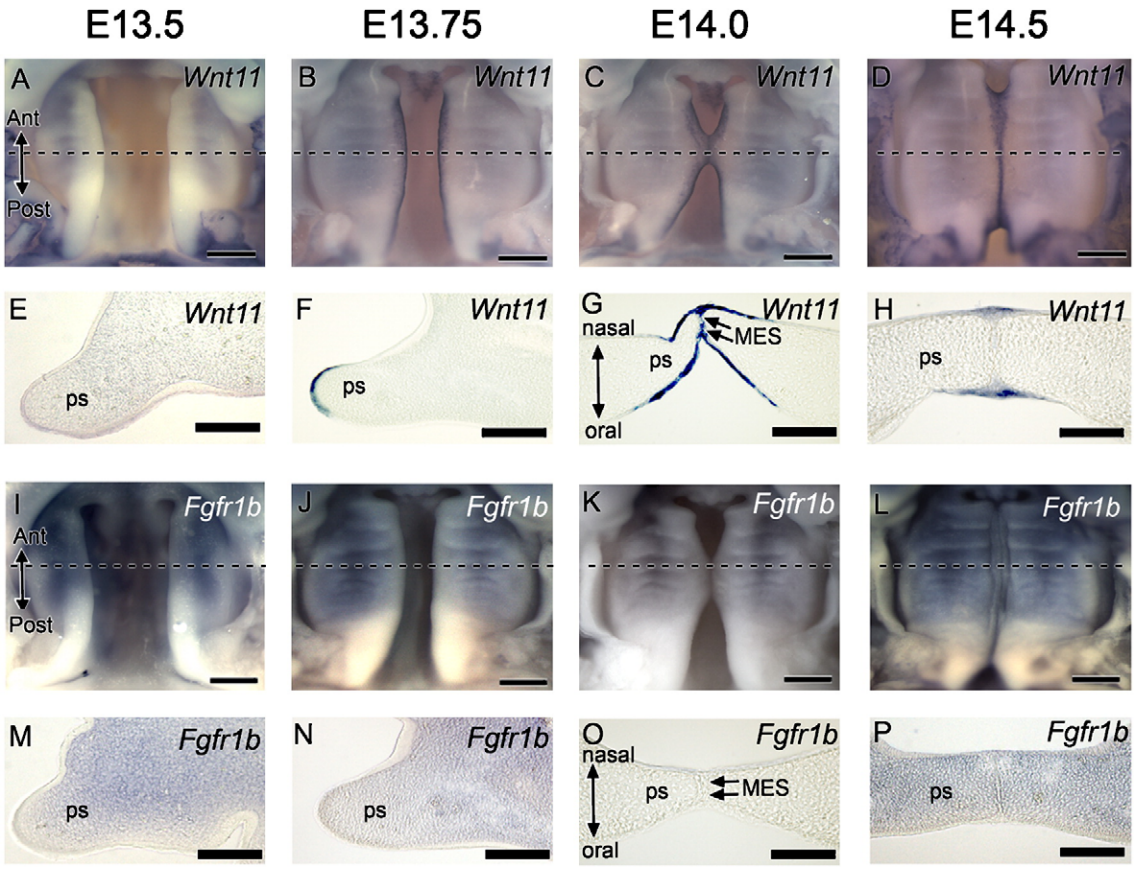

Fig. 1. (A–P) *Wnt11* and *Fgfr1b* expressions are detected by whole-mount *in situ* hybridization and sectioning. Only frontal sections are shown. (A, E) At E13.5, *Wnt11* is expressed weakly in the posterior region but not in the middle region of the palatal shelf. (B, F) At E13.75, *Wnt11* is expressed along the MEE region, with strong expression in the palatal epithelium. (C) At E14.0, *Wnt11* is expressed in the MEE region and contact area between the palatal shelves. (G) *Wnt11* is strongly expressed in both the MEE (arrows) and MES. (D) At E14.5, *Wnt11* expression is restricted along the fusion line of the palate. (H) *Wnt11* is expressed in the palatal epithelium on both the oral and nasal sides of the palate. (I, J) *Fgfr1b* is expressed in the anterior to middle regions at E13.5 and E13.75. (M, N) *Fgfr1b* is expressed in the palatal mesenchyme. (K) After the initiation of palatal contact at E14.0, *Fgfr1b* is expressed in the anterior to middle regions of the palate. (O) There is no *Fgfr1b* expression in the region of palatal contact in either the epithelium or mesenchyme. (L) At 14.5, *Fgfr1b* is expressed from the anterior to posterior regions of the palate. (P) *Fgfr1b* expression is strong in the middle region of the palatal mesenchyme. ant, anterior; black dotted line, section plane; post, posterior; ps, palatal shelf; scale bars: A–D, I–L, 500 μm; E–H, M–P, 200 μm.

detected in both the palatal epithelium and mesenchyme on the Wnt-11 overexpressed domain compared to the control domain (Fig. 3A). In addition, TUNEL-positive cells were observed in the epithelium of the midline region, indicating fusion of the two developing palates. On the Wnt-11 overexpressed side, strong positive reactions for cell death were identified in the epithelium and underlying mesenchyme (Fig. 3C), while few TUNEL-positive cells were observed on the control side (Fig. 3D). The vector control showed that TUNEL-positive cells were not altered at E13.5 (data not shown). On the other hand, the number of PCNA-positive cells differed between the experimental and control sides after 48 h (Fig. 3G). The number of PCNA-positive was clearly lower in the Wnt-11 overexpressed region than in the control (Figs. 3I and J). To determine whether Fgf signaling is involved in the maintenance and/or induction of cell death and in proliferation in secondary palate development, beads soaked in SU5402 (10 mM) were implanted on one side of the palatal shelf and cultured for 48 h (DMSO as the control), and TUNEL and PCNA were applied to the developing palate (Figs. 3B and H). SU5402 altered the number of apoptotic cells (Fig. 3B). After SU5402 beads implantation at E13.5, the number of TUNEL-positive cells was clearly higher than in the control after 48 h (Figs. 3E and F). SU5402 also altered cell proliferation (Fig. 3H). The number of proliferating cells was lower around SU5402-soaked beads than in the control during palate development (Figs. 3K and L). The numbers of TUNEL- and PCNA-positive cells were not altered in the control after implanting DMSO-soaked beads (data not shown).

Inhibition of apoptosis by z-VAD-fmk treatment disrupted palate fusion

In order to investigate the effect of z-VAD-fmk treatment as an inhibitor of apoptosis, developing palates were cultured in media containing 25 μM, 50 μM, or 100 μM z-VAD-fmk for 48 h. H–E staining of serial frontal sections revealed differences between the DMSO controls and z-VAD-fmk-treated specimens (Figs. 4A–C). In the controls, developing pairs of palates were completely fused after 48 h (n=9/10) (Fig. 4A). The fate of the MES in the experimental group clearly differed with the dose,

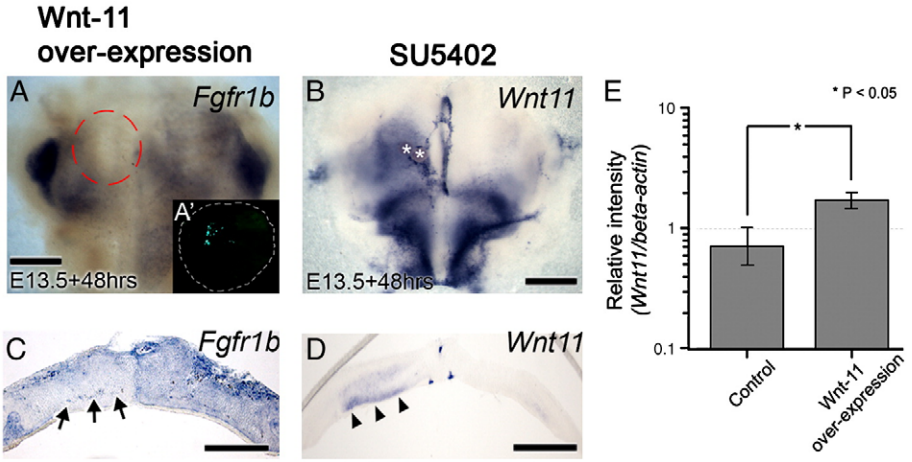

Fig. 2. (A, C) Overexpressed Wnt-11 alters *Fgfr1b* expression pattern. Electroporation is applied between the anterior and middle regions of the palate (red dotted circle). All specimens are electroporated at E13.5 and then incubated for 48 h. Wnt-11 is successfully transfected, as indicated by the GFP expression (A′). After Wnt-11 overexpression, *Fgfr1b* expression is downregulated in these regions (arrows). (B, D) SU5402-soaked beads (10 mM, asterisk) are implanted into the palate at E13.5 and the specimens are then incubated for 48 h. Expression of *Wnt11* is upregulated strongly in the epithelium and weakly in the mesenchyme by the implantation of SU5402-soaked beads (arrowheads). (E) Expression levels of *Wnt11* are examined by RT-qPCR in the palate at E13.5 after 48 h of culturing. *Wnt11* expression levels increase dramatically after Wnt-11 overexpression relative to control groups. $*P<0.05$ as determined by ANOVA; asterisk, bead; red dotted circle, DNA injection region; white dotted line, boundary of the cultured palate; scale bars: A, B, 500 μm; C, D, 100 μm.

with z-VAD-fmk at a low concentration (25 μM) not inhibiting palatal fusion ($n=11/14$), with some MES cells remaining (arrowheads in Fig. 4B), while MES cells still remained (arrowheads in Fig. 4C) with z-VAD-fmk at a high concentration (50 μM) ($n=12/14$) (Fig. 4C), with similar results for 100 μM z-VAD-fmk ($n=11/11$) (data not shown).

The number of TUNEL-positive cells was counted in a 100 μm^2 region of both control and z-VAD-fmk-treated specimens. The number of apoptotic cells was significantly lower after z-VAD-fmk treatment than in the DMSO control (Figs. 4D–F, and data not shown), with the difference increasing with the concentration of z-VAD-fmk. There were 8.7 TUNEL-positive cells/100 μm^2 in the palate of DMSO control specimens (Fig. 4D, and data not shown), but 4.6 cells/100 μm^2 after treatment with 25 μM z-VAD-fmk. However, palatal shelves were fused with the MES particles (arrowheads in Fig. 4B) only on the oral and nasal sides (Figs. 4B and E, and data not shown). Moreover, there were only 2 cells/100 μm^2 in specimens treated with 50 μM or 100 μM z-VAD-fmk (Fig. 4F, and data not shown).

Wnt11 knockdown using siRNA

To determine the function of *Wnt11* during palatogenesis, we performed a gene knockdown investigation using *Wnt11* siRNA (siRNA ID 65306, Ambion). *Wnt11* siRNA was applied at 500 nM to palatal shelves at E13.5, which were then cultured for 72 h. After *in vitro* culture, we performed H–E staining to identify alterations to the pattern of palatogenesis (Figs. 5A–C). There were no morphological differences between the control siRNA treatment group and the control group ($n=13/15$) (Figs. 5A and B), with the MES completely disappearing and the palatal shelves fusing. However, there were morphological differences between the *Wnt11* siRNA treatment group and the control siRNA treatment group (Figs. 5B and C). After treatment with 500 nM *Wnt11* siRNA, the MES was still detected in the midline of the palate ($n=15/15$) (arrowheads in Fig. 5C). In order to understand the precise relationship between *Wnt11* and apoptosis, we performed TUNEL staining after treatment with *Wnt11* siRNA (Figs. 5D–F). In control and control siRNA, apoptotic bodies decreased with the disappearance of the MES (Figs. 5D and E). As expected, *Wnt11* knockdown by siRNA inhibited apoptosis in the remaining MES (Fig. 5F). RT-qPCR was used to examine the effectiveness of the siRNA system. Significant differences were found after *Wnt11* siRNA treatment. The level of *Wnt11* was lower in the 500 nM *Wnt11* siRNA treatment group than in the control and control siRNA treatment groups ($n=24$) (Fig. 5G). In order to confirm the relationship between *Wnt11* and *Fgfr1b* in palatogenesis, we used RT-qPCR to evaluate the expression level of *Fgfr1b* after *Wnt11* siRNA treatment. The expression level of *Fgfr1b* was dramatically higher in the *Wnt11* siRNA treatment group than in the control and control siRNA treatment groups ($n=15$) (Fig. 5H).

Discussion

Expression patterns of Wnt11 and Fgfr1b in the developing secondary palate

Convergent extension has been examined during palatogenesis when MEE cells intercalate to the MES during palatal fusion (Tudela et al., 2002). Convergent extension as regulated by *Wnt11* is also critical to the formation of the vertebrate body axis (Jopling and den Hertog, 2005). The relationships between convergent extension and the *Wnt11* and *Fgfr1* genes in gastrulation cell movements have been reported (Chung et al., 2005; Jopling and den Hertog, 2005). In addition, *Wnt11* and

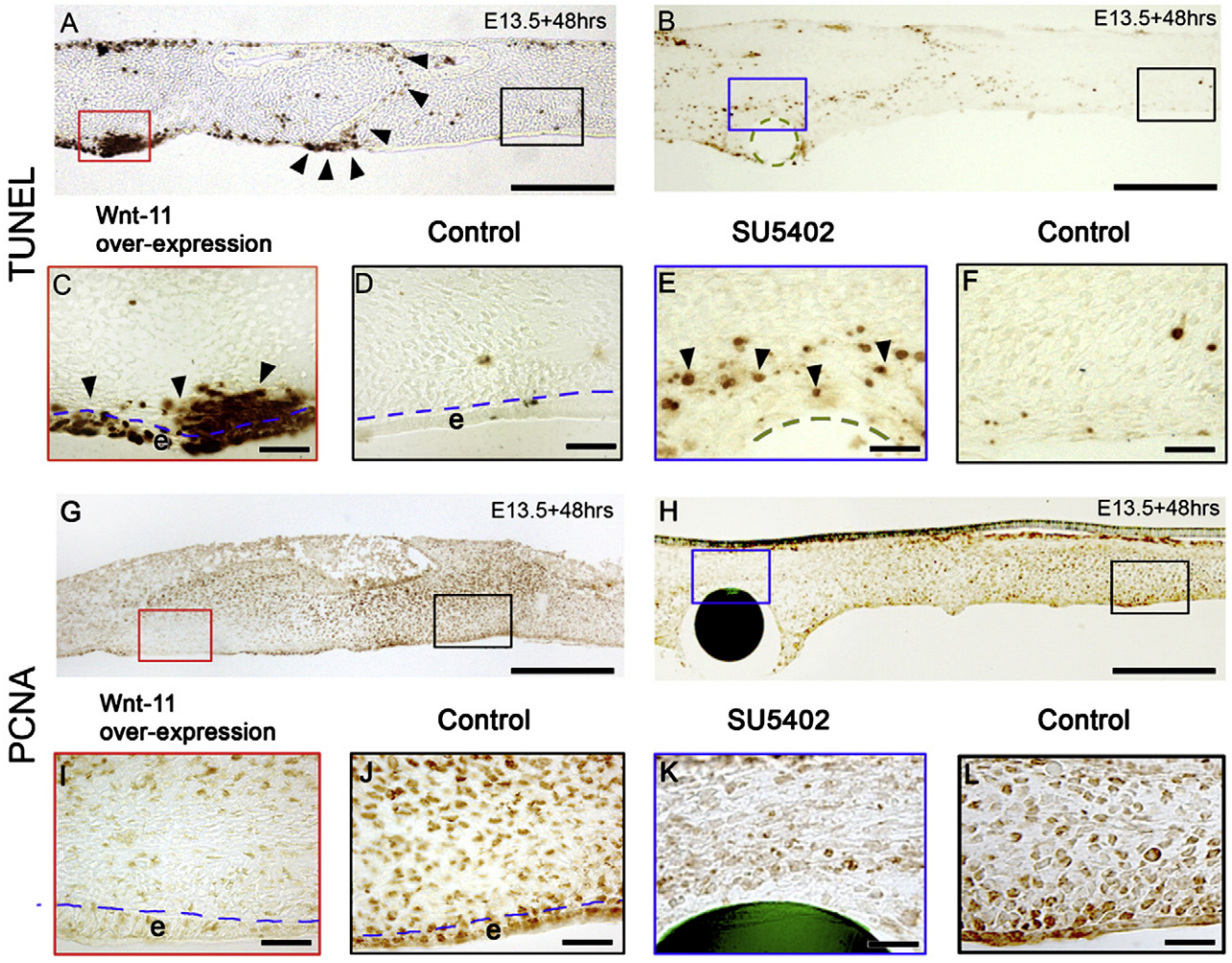

Fig. 3. (A, G) TUNEL and PCNA assay on frontal sections after overexpression studies in cultured palates (at E13.5+48 h). (A, C, D) The number of TUNEL-positive cells increased markedly after Wnt-11 overexpression. (A) TUNEL-positive cells are detected in the epithelium of the fusion region (arrowheads). (C) Overexpressed Wnt-11 induces apoptosis in the epithelium and in the mesenchyme beneath the epithelium (arrowheads), but not on the control side. (D) A few apoptotic cells are detected on the control side. (G, I, J) Overexpressed Wnt-11 alters the cell proliferation rate in the cultured palate relative to the control side. (I, J) There are fewer PCNA-positive cells on the Wnt-11 overexpressed side than on the control side. (B, H) Cell death and proliferation are examined after implanting SU5402-soaked beads (10 mM) for 48 h. (B) SU5402 alters the cell death rate during palate development. (E) Apoptotic cells are induced near the SU5402 beads (green dotted circle). (F) A few TUNEL-positive cells are evident on the control side. (H) Implanting SU5402-soaked beads altered the number of PCNA-positive cells. (K, L) SU5402-soaked beads decreased the number of PCNA-positive cells relative to the control side. Blue dotted line, border of epithelium and mesenchyme; e, epithelium; green dotted circle, bead; scale bars: A, B, G, H, 200 μm; C–F, I–L, 20 μm.

Fgfr1b play essential roles in vertebrate development, including the branching morphogenesis in the kidney of the mouse (Majumdar et al., 2003; Chi et al., 2004; Tao et al., 2005). In the present study, we hypothesized that *Wnt11* and *Fgfr1b* were involved in palatogenesis, whose cellular mechanisms are similar to convergent extension. While *Wnt11* and *Fgfr1b* play important roles in convergent extension (Trokovic et al., 2003), the precise molecular interactions between *Wnt11* and *Fgfr1b* underlying palatogenesis have not been elucidated. In order to understand the function of *Wnt11* and *Fgfr1b*, we examined gene expression patterns between E13.5 and E14.5 using *in situ* hybridization since these developmental stages are critical to palate formation. Prior to fusion, the expression patterns of *Wnt11* in the margin of the palatal shelf epithelium suggested that *Wnt11* is involved in the fusion of the palatal shelves. In contrast, *Fgfr1b* was expressed in the developing palatal mesenchyme from the anterior to posterior regions. Particularly at E14.0, when palatal contact is initiated, *Fgfr1b* was not expressed in either the palatal epithelium or mesenchyme along the palatal margin (Fig. 1). These temporal expression patterns of *Wnt11* and *Fgfr1b* during palate development suggest that they play important roles through interactions or cascade pathways.

Wnt11 and Fgfr1b modulate the cellular events during palatogenesis

To clarify the molecular interactions between *Wnt11* and *Fgfr1b* during palatogenesis, Wnt-11 overexpression – representing an enhancement of the function of *Wnt11* – was examined using an electroporation method. The results clearly showed that the expression of *Fgfr1b* was downregulated in the

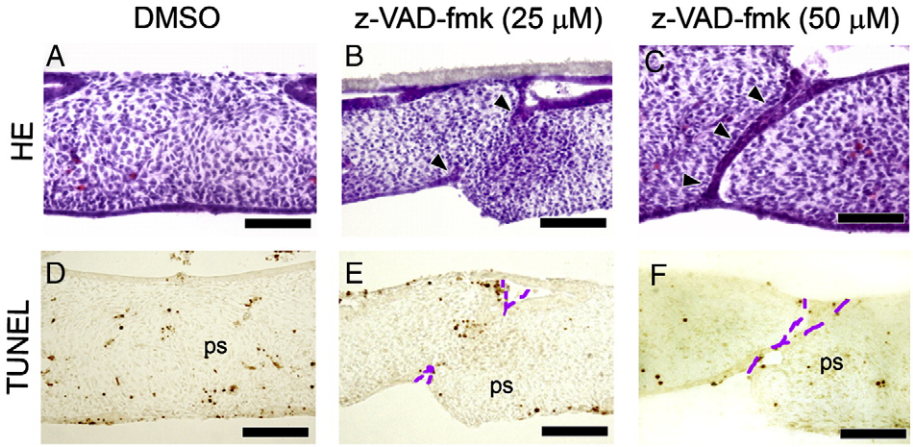

Fig. 4. (A–F) H–E and TUNEL staining are examined after applying 25 μM or 50 μM z-VAD-fmk. (A) After 48 h in culture, the two palatal shelves are completely fused in the DMSO control group. (B) After treatment with 25 μM z-VAD-fmk, the two palatal shelves are fused with the MES particle (arrowheads) only on the oral and nasal sides. (C) Palatal shelves are not fused with the MES in specimens treated with 50 μM z-VAD-fmk (arrowheads). (D–F) The number of TUNEL-positive cells is significantly lower in z-VAD-fmk-treated specimens than in the control group cultured with DMSO. (D) More apoptotic cells are detected in DMSO controls than in z-VAD-fmk treatment. (E) The number of apoptotic cells is reduced after treatment with 25 μM z-VAD-fmk relative to the control group. (F) Few apoptotic bodies are detected in the palate in specimens treated with 50 μM z-VAD-fmk. Purple dotted line, remaining MES; ps, palatal shelf; scale bars: A–F, 100 μm.

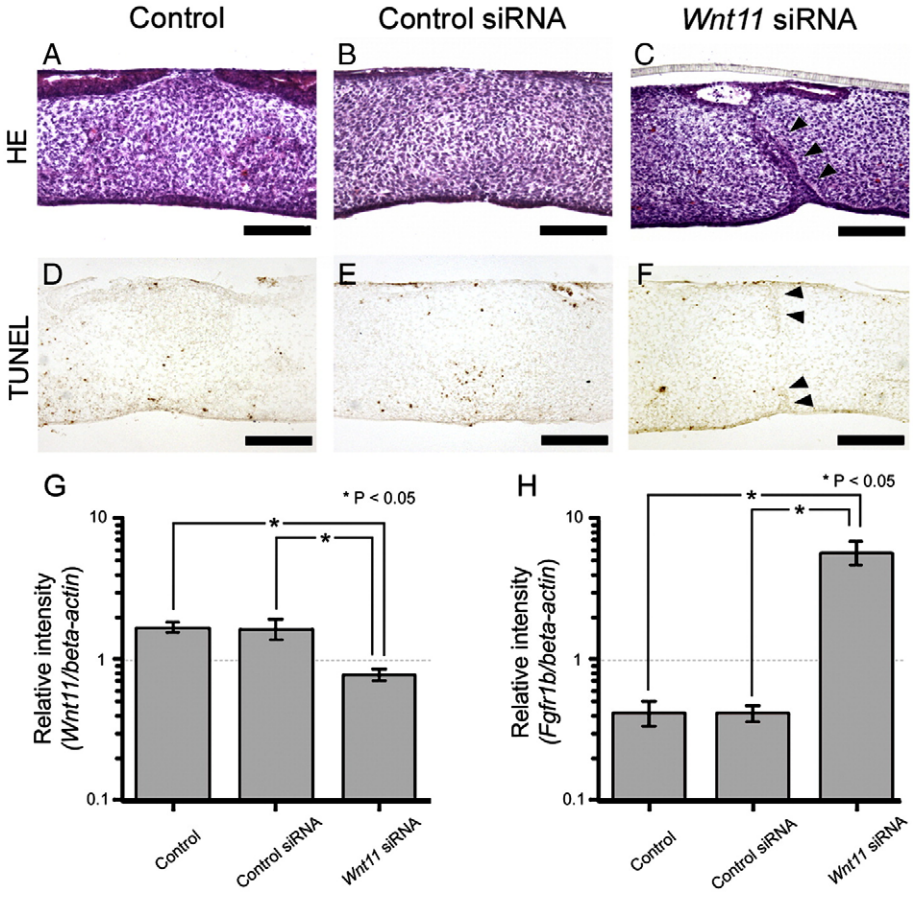

Fig. 5. (A–F) H–E and TUNEL staining are examined after 500 nM siRNA treatment. (A, B) The palatal shelves are completely fused in the control and control siRNA treatment groups. (C) However, the MES remained (arrowheads) and the palatal shelves are not fused in the group treated with 500 nM *Wnt11* siRNA. (D–F) TUNEL staining revealed no significant positive reaction of apoptosis in the MES region both in the control and siRNA treatment groups. (D and E) Apoptotic bodies are reduced with the disappearance of the MES. (F) There is no positive TUNEL staining in the remaining MES region. (G) *Wnt11* expression level is detected in cultured palate (at E13.5+72 h). The level of *Wnt11* expression is significantly lower after treatment with 500 nM *Wnt11* siRNA than in the control and control siRNA treatment groups. (H) Expression levels of *Fgfr1b* are examined in the cultured palate (at E13.5+72 h). *Fgfr1b* expression levels are dramatically increased after *Wnt11* siRNA treatment relative to the control and control siRNA treatment groups. *$P<0.05$ as determined by ANOVA; arrowheads, remaining MES; scale bars: A–F, 100 μm.

developing palate (Fig. 2). Moreover, knockdown using *Wnt11* siRNA induced the intensity of *Fgfr1b* (Fig. 6H), which suggests that a specific pathway of *Wnt11* regulates *Fgfr1b* during palatogenesis. *Wnt11* expression was upregulated by the implantation of SU5402-soaked beads, concomitant with the loss of function of Fgfr1 (Fig. 2). Thus, mesenchymal expression of *Fgfr1b* would negatively regulate the epithelial expression of *Wnt11*. These altered expression patterns of *Wnt11* and *Fgfr1b* suggest that *Fgfr1b* and *Wnt11* negatively regulate each other during palatogenesis.

Based on the specific expression patterns of *Wnt11* in the MEE and MES during palatogenesis, we hypothesized that *Wnt11* is involved in palatal fusion, which is induced by apoptosis. Recent studies have shown that apoptosis plays important roles in palatal fusion (Vaziri Sani et al., 2005) and that members of the Wnt family regulate cell death both positively and negatively (Lobov et al., 2005). In order to understand the function of *Wnt11*, electroporation was performed for overexpression of Wnt-11 away from the MEE. Naturally, *Wnt11* was expressed in the MEE and MES regions of the epithelium (Fig. 1). Apoptotic cells were also detected in the MES where *Wnt11* expressed region (Fig. 3). We therefore attempted to avoid overlap between normal *Wnt11* expressions and overexpressed Wnt-11, injecting the Wnt-11 expression construct away from the MEE. TUNEL staining after Wnt-11 overexpression revealed that *Wnt11* modulates cell death in palatogenesis. Inhibition of Fgfr1 by SU5402 altered cell proliferation and apoptosis in the developing palate, suggesting that the Fgfs influence the cellular events including cell proliferation and apoptosis in palate development either directly or indirectly. These results are consistent with previous studies that have provided evidence of molecular interactions between Fgf and Fgfrs in palatogenesis (Garcia-Maya et al., 2006; Ziv et al., 2006).

These results suggest that cell proliferation – which is reported to be closely related to the elevation of the developing palate – would be regulated in part by *Fgfr1b*, whereas *Wnt11* potentially regulates palatal fusion by mediating cell death. Furthermore, the relationship between *Fgfr1b* and *Wnt11* is required for correct processes of palatal development, such as proliferation and fusion induced by apoptosis.

Apoptosis for palatal fusion is modulated by molecular interactions between Wnt11 and Fgfr1b

Vaziri Sani et al. (2005) reported that the fate of the MES during palatal development is still controversial. Previous studies showed that apoptosis eliminates some MEE cells, which results in the correct migration and differentiation during palatal fusion (Mori et al., 1994; Taniguchi et al., 1995; Cuervo and Covarrubias, 2004). However, Takahara et al. (2004) used a palate organ culture with apoptosis inhibitors to demonstrate that cell death is not required for mammalian palatogenesis. Moreover, there is ultrastructural and molecular evidence of EMT in the disappearing MES cells (Martýnez-Alvarez et al., 2000), although more recent *in vivo* genetic fate-mapping results showed that the occurrence of EMT is mutually contradictory during palatal fusion (Vaziri Sani et al., 2005; Jin and Ding, 2006).

Based on our *in vitro* Wnt-11 overexpression results, we suggest that *Wnt11* induces apoptosis in the palatal epithelium and mesenchyme through inhibition of *Fgfr1b*, which plays important roles in cell proliferation. However, Wnt-11 overexpression was not sufficient to elucidate function of apoptosis in palatal fusion. We examined the precise role of apoptosis in palatal fusion by treating samples with the pancaspase inhibitor z-VAD-fmk, which blocked the apoptosis induced by p21 (Wood and Newcomb, 1999) (Fig. 4). The H–E staining results suggest that treatment with z-VAD-fmk at a concentration greater than 50 μM disrupted palate fusion by decreasing the number of apoptotic cells.

Based on previous studies (Shiomi et al., 2006; Nakajima et al., 2007), siRNA treatment was used in the present study to examine the functional significance of *Wnt11* in regulating palatal fusion (Fig. 5). Majumdar et al. (2003) reported that *Wnt11* mutant mice showed various problems, such as a smaller kidney and ureteric and kidney branching defects. However, cleft palate has not been reported in *Wnt11* transgenic mice. In the present study, we treated with *Wnt11* siRNA at E13.5 for

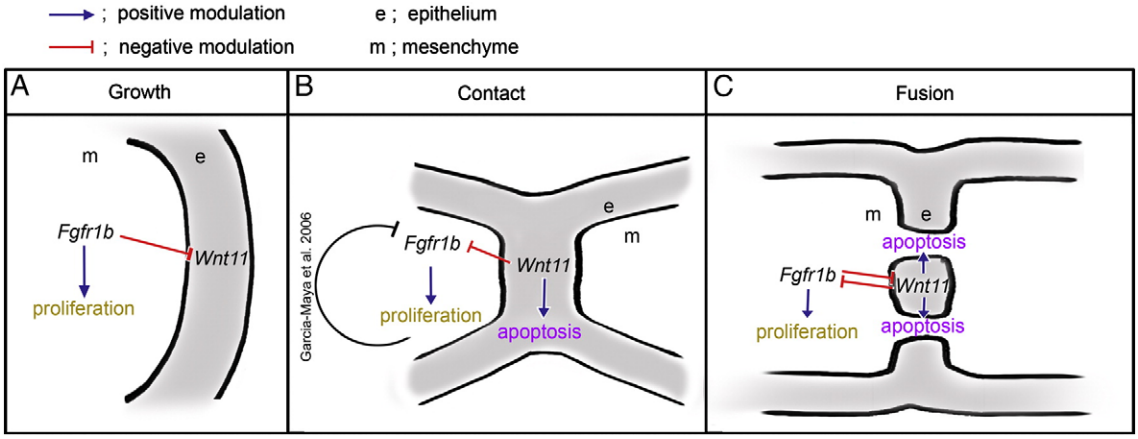

Fig. 6. The molecular interactions between *Wnt11* and *Fgfr1b* modulate various cellular events including cell proliferation and apoptosis to ensure correct development. Black blunt arrow, previous finding; blue arrow, positive modulation; e, epithelium; m, mesenchyme; red blunt arrow, negative modulation.

72 h. One advantage of this system is that it may allow the examination of gene regulation in a time- and tissue-specific manner. Our use of this *in vitro* culture system with *Wnt11* siRNA confirmed that *Wnt11* was closely related to palatal fusion. As we hypothesized, knockdown of *Wnt11* resulted in the failure of palatal fusion, which also occurred after treatment with z-VAD-fmk. After *in vitro* culture at E13.5 for 72 h, palatal shelves were completely fused with elimination of the MES via apoptosis (Figs. 3A, 5D and E). However, after *Wnt11* siRNA treatment, apoptotic cells were not detected even when the MES did not disappear (Fig. 5F). These results suggest that *Wnt11* is a key factor for palatal fusion through the induction of apoptosis.

Molecular interactions govern cellular modulations in palatogenesis

Our results appear to demonstrate that cellular modulations controlled by molecular interactions between *Wnt11* and *Fgfr1b* are important to the correct development of the palate in three critical phases during palatogenesis. In the first (growth) phase between E13.5 and E14.0, *Fgfr1b* induce cell proliferation and inhibit *Wnt11* expression (Fig. 6A). Inhibitory feedback loops downstream of FGF signaling factors regulate signal propagation via a MAP kinase cascade downstream of FGF receptor activation (Eblaghie et al., 2003). In the second (contact) phase at E14.0, negative feedback of Fgf-induced cell proliferation would gradually inhibit *Fgfr1* expression over time (Garcia-Maya et al., 2006), which might in turn cause the gradual expression of *Wnt11*, inducing apoptosis in the adjacent epithelium (Fig. 6B). In the third (fusion) phase at E14.5, *Wnt11*-induced apoptosis in the epithelium (MES) would be eliminated over time, resulting in *Fgfr1b* being gradually expressed for growth of the fused palate (Fig. 6C). The results of z-VAD-fmk treatment revealed that *Wnt11*-mediated apoptosis is closely related to palate fusion in this third phase. Interactions between *Wnt11* and *Fgfr1b* could represent a pivotal cellular-control mechanism for achieving the precise morphogenesis of the secondary palate. The model is steady with the data at hand, but given the rapid advancing of the field, no doubt the model will need some dynamic remodeling over time as additional pathways and molecular cross-talk emerge.

An understanding of the unique developmental processes underlying palatogenesis will help to elucidate the fundamental molecular mechanisms underlying cleft palate. We propose that Wnt/Fgfr cross-talk plays an important, cell-autonomous role in regulating the fate of MEE cells during palatogenesis in both mice and humans. Future studies should examine the factors that play fundamental roles in mediating palate development using experimental model system, such as microarray, to understand the precise molecular mechanisms underlying palatal development.

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